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RESEARCH****Research Report****A novel neurotrophic therapeutic strategy for experimental stroke**

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ABSTRACT

Human chorionic gonadotropin (hCG) promotes proliferation of endogenous neural stem cells, and erythropoietin (EPO) promotes differentiation of these cells into neural stem cells. The current study examined effects of sequential administration of these two compounds, initiated 24 h after stroke. At that time, rats were randomized into four treatment groups: hCG+EPO (3 IM doses hCG over 5 days, followed by 3 IV doses EPO over 3 days), hCG+Saline using the same schedule, Saline+EPO using the same schedule, or neither drug (Saline+Saline). The primary endpoint was the composite neurological score, measured 11 times, from 1 h until 12 weeks post-insult. The neurological score was different across treatment groups ($p < 0.03$). Pairwise testing of groups found that the hCG+EPO group had significantly better behavior at 6/10 post-stroke time points as compared to Saline+Saline. The differences observed when comparing the two-drug group with placebo were less apparent when comparing either of the one-drug groups with placebo. The two one-drug treatment arms did not significantly differ at any time point. Treatment with hCG+EPO significantly reduced total lesion volume by 82–89% compared to the other three treatment groups. The current therapeutic strategy improved behavioral outcome and reduced lesion volume with a time window of 24 h after the onset of stroke. The results from these experiments provide new insight into the effects of these two growth factors on stroke in rats, and could suggest a potential for translation into human stroke studies.

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1. Introduction

For most patients with acute ischemic stroke, limited therapeutic options exist beyond 8 h after the onset of stroke. Even with the acute reperfusion therapies currently available, most patients who are treated still show significant signs of long-

term disability. Consequently, additional therapeutic strategies are needed. One set of therapies that might be of value in this regard aims to promote repair by administration of growth factors.

Many growth factors show spontaneously increased levels in the weeks following stroke (Finklestein et al., 1990). A

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number of different growth factors have been found to improve the long-term outcome in animal models when administered the first few days after stroke (Kawamata et al., 1997; Kawamata et al., 1998; Schneider et al., 2006; Wang et al., 2004). One mechanism by which growth factors might influence outcome after stroke is by their actions on endogenous neural stem cells. Human chorionic gonadotropin (hCG), like other neurotrophic factors (Craig et al., 1996; Shingo et al., 2001; Kolb et al., 2007), promotes proliferation of endogenous neural stem cells. hCG resides in the same growth factor family as nerve growth factor (Lei and Rao, 2001), crosses the blood brain barrier (Lukacs et al., 1995), and normally has its receptor present in adult rat brains (Al-Hader et al., 1997). Erythropoietin (EPO) is a growth hormone (Chen and Chopp, 2006) that also crosses the blood brain barrier (Ehrenreich et al., 2002; Kaushansky 2006) and its receptor mRNA is readily detectable in central nervous system neurons and glia (Digicaylioglu et al., 1995). EPO also is known to promote the differentiation of endogenous neural stem cells into neural stem cells (Shingo et al., 2001).

The current study builds directly on a prior report by Kolb et al. (2007), who found improved behavioral outcome and reduced infarct volume with delayed administration of intraventricular epidermal growth factor followed by EPO (Kolb et al., 2007) in rats with experimental stroke. We changed to hCG+EPO because there is more human experience with hCG, and both are thought to be endogenous stem cell mitogens. We decided first to use hCG to promote proliferation and then use EPO to promote survival and differentiation of new progenitors. This treatment paradigm (hCG followed by EPO) was initiated 24 h after experimental stroke, which has increased human translational potential.

2. Results

2.1. Physiological variables

There were no significant differences with respect to rectal and cranial temperatures between groups. All animals had reduced body weight during the first week, but regained weight afterward. There were no significant differences in body weight between groups at any point during the 12-week survival period.

2.2. Behavioral findings

Composite Neurological Scores, the primary behavioral endpoint, varied according to treatment. Neurological score was normal (0) in all animals before MCAo. Contralateral forelimb placing deficits were clearly present at 60 min after MCAo in all groups (score, 10–11). Three animals were excluded based on inadequate ischemia (score, 8–9). The main effect of treatment was significant ($F_{3,16}=4.1$, $p<0.03$), as was main effect of time ($F_{10,7}=23.7$, $p=0.0002$); the time \times group interaction across the four treatment groups approached significance ($p=0.0554$, repeated-measures ANOVA, Fig. 1). Post-hoc comparison of the two-drug treatment group (hCG+EPO) vs. placebo (Saline–Saline) group found that scores in the two-drug group were significantly lower ($p<0.05$, Fisher's pro-

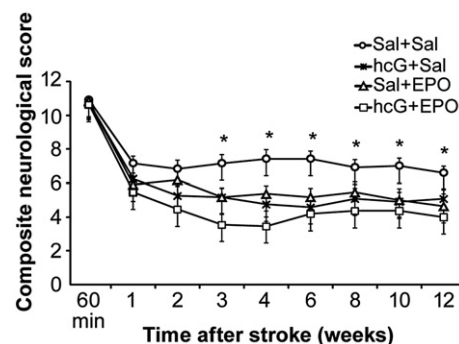


Fig. 1 – Improvement in the primary behavioral outcome measure is shown over time for the four treatment groups. Animals receiving hCG+EPO, as compared with Saline+Saline, had significantly lower scores at multiple timepoints. Data are mean \pm SEM. * $p<0.05$, Saline+Saline vs. hCG+EPO.

tected least-significant difference test), indicating better neurological status, at 6 of the 10 post-stroke time points: 3 wk, 4 wk, 6 wk, 8 wk, 10 wk, and 12 wk after middle cerebral artery occlusion (MCAo).

The differences observed when comparing the two-drug group with placebo were much less apparent when comparing either of the one-drug groups with placebo. Thus, when comparing the neurological scores for Saline+EPO vs. Saline+Saline, scores in the active treatment group were significantly lower at only 5/10 timepoints, i.e., at 3 wk, 4 wk, 6 wk, 10 wk, and 12 wk post-MCAo. Similarly, when comparing the neurological scores for hCG+Saline vs. Saline+Saline, scores in the active treatment group were again significantly lower at fewer (7/10) timepoints, i.e., at 2 wk, 3 wk, 4 wk, 6 wk, 8 wk, 10 wk, and 12 wk post-MCAo.

Consistent with the above results, direct comparisons of the two-drug with the one-drug group favored the former. Thus, when comparing the composite neurological scores for hCG+EPO vs. Saline+EPO, scores in the two-drug group were significantly lower ($p<0.05$) at 24 h and 4 weeks post-stroke. At all the other time points, mean scores for hCG+EPO were always lower than Saline+EPO, but these did not reach significance. When comparing neurological scores for hCG+EPO vs. hCG+Saline, scores in the two-drug group were lower in all but one timepoint, but again these did not reach significance. The two one-drug treatment arms were not significantly different at any timepoint.

Regarding the secondary behavioral endpoints, adhesive test scores also varied according to treatment. The main effect of treatment was significant ($F_{3,42}=2.8$, $p<0.05$), as was the main effect of time ($F_{7,36}=7.3$, $p<0.0001$); the time \times group interaction across the four treatment groups again approached significance ($p<0.08$). Post-hoc comparison found that at 3/8 timepoints, all three active treatment groups showed significantly better adhesive scores as compared to the Saline+Saline group. Swimming test scores showed no significance for main effect of treatment group or for the time \times group interaction, though the main effect of time ($F_{7,33}=3.8$, $p<0.005$) was significant. The cylinder test scores showed no significant main effect of treatment

group or time, though the time×group interaction showed a trend ($p=0.12$) toward significance.

2.3. Histopathology

Lesion volume was significantly different across groups ($p<0.03$, ANOVA, Fig. 2). Post-hoc testing between groups disclosed that the lesion volume for hCG+EPO was 82% lower than the Saline+Saline group ($p<0.008$), 86% lower than the Saline+EPO group ($p<0.004$), and 89% lower than the hCG+Saline group ($p<0.009$). Lesion volume for the Saline+EPO and for the hCG+Saline groups did not differ significantly from each other, and neither differed from Saline+Saline. Percent tissue loss showed very similar trends, but ANOVA did not reach significance (hCG+EPO: 12%; hCG+Saline: 42%; Saline+EPO: 35%; Saline+Saline: 32%).

In three groups (the Saline+EPO, hCG+Saline, and Saline+Saline treated animals), microscopic examination of the brain confirmed a large extent of cystic necrosis involving neocortex that extended to subcortical structures. However, in brains from animals treated with hCG+EPO, frank cystic necrosis in the neocortex was present in only 2 of 10 animals. In the other

eight brains, the neocortex was structurally intact but exhibited subtle to prominent hypercellularity attributable to increases in microglial nuclei; however, no apparent necrotic or obvious apoptotic neurons were observed.

Distribution of lesion areas at 12 weeks after MCAO is presented in Fig. 3. Treatment with hCG+EPO significantly reduced total lesion areas at two bregma levels compared to hCG+Saline treated groups.

Seven animals died during the experiment: two rats in the hCG+EPO group, three rats in the hCG+Saline group, one rat in Saline+EPO and one rat Saline+Saline groups. All animals died during one week after surgery.

3. Discussion

The current study aimed to evaluate a new treatment paradigm: use of two sequentially administered growth factors — one to promote endogenous neural stem cell proliferation (hCG) and a second to promote differentiation of these cells into neural stem cells (EPO) (Shingo et al., 2001). The two-drug regimen, initiated 24 h after the onset of stroke, showed significant improvement in the primary behavioral measure and reduction of lesion volume. The current results achieved these aims in several ways. For example, the two-drug regimen was superior to the placebo; note that this was true more often than either one-drug group being superior to the placebo. Also, lesion volume was substantially reduced with the two-drug regimen, but not with either one-drug regimen.

Brain repair represents a potential avenue to reduce disability after stroke with treatments initiated beyond the current eight-hour time window (Cramer, 2008). A number of therapeutic approaches have been advanced. One of them is EPO, a hematopoietic growth hormone, which regulates survival, proliferation and differentiation of erythroid progenitor cells (Shingo et al., 2001). In preclinical studies, treatment with EPO at 24 h after onset of stroke significantly improved functional outcome (Wang et al., 2004). Although EPO seems to be potentially safe at the neuroprotective-proven doses, cardiovascular or cerebrovascular events can occur as a result of its bone marrow stimulating activities. EPO therapies for anemia or cerebrovascular diseases require frequent injections or high-dose systemic administration that may cause unwanted side effects. Hypertension, thrombosis and increased hematocrit are common side effects of EPO (Chen and Chopp, 2006). High dose EPO (5,000–10,000 U/kg) is necessary for achieving neuroprotection in focal cerebral ischemia in rats (Chang et al., 2008).

Kolb et al. (2007) found that, in a rat model of stroke, sequential intraventricular administration of epidermal growth factor, followed by erythropoietin, promoted improved behavioral status as well as regeneration of damaged neocortex. These effects were seen with their two-drug regimen, but not with administration of only one of their two growth factors. Favorable effects were seen even when growth factor administration was delayed for up to seven days after stroke. The study by Kolb et al. (2007) used intraventricular drug administration, which is challenging in human subjects, and

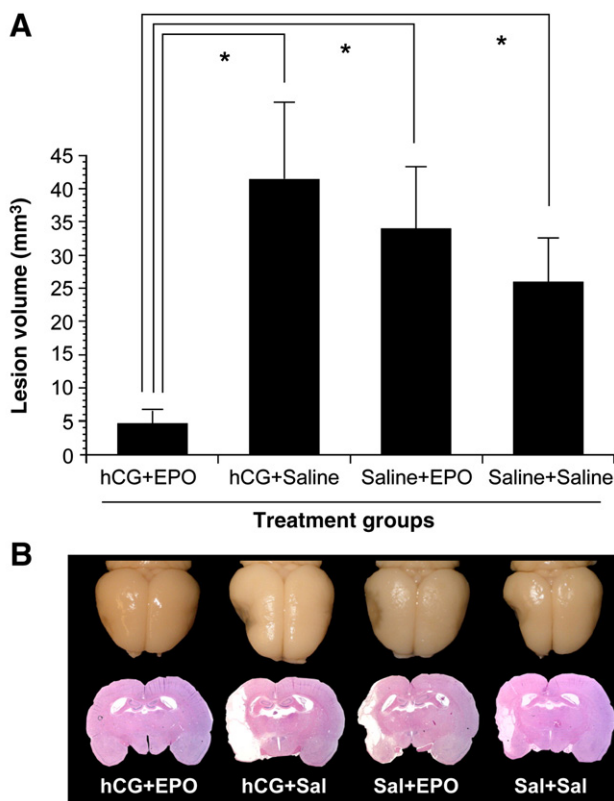


Fig. 2 – Panel A: Lesion volume at week 12 in animals with treatment starting 24 h after stroke. As compared to animals receiving hCG+EPO, the lesion volume was significantly smaller ($*p<0.01$) in animals receiving one (hCG+Saline or Saline+EPO) or no (Saline+Saline) drug. Data are mean±SEM. **Panel B:** The whole brain (top row) and a coronal slice (at bregma level –1.8 mm) are consistent with these differences in lesion volume.

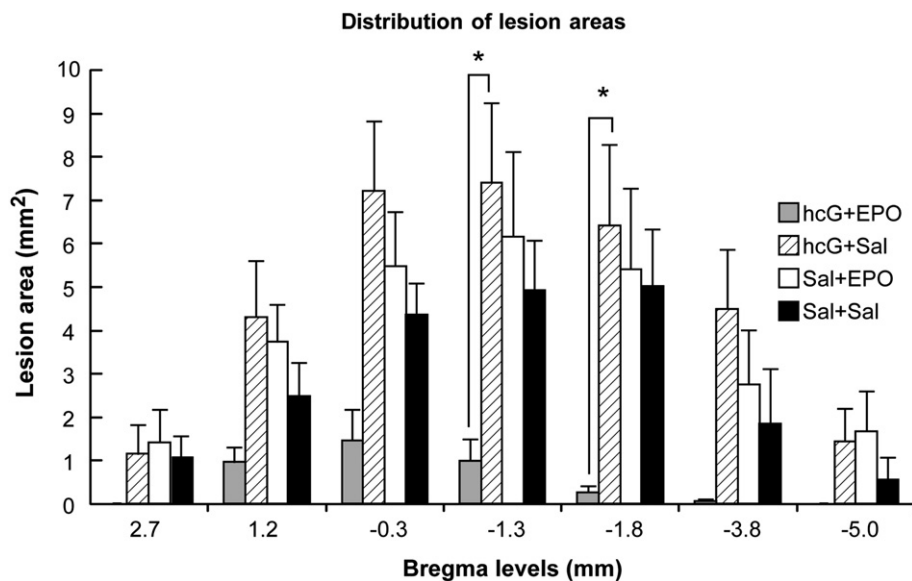


Fig. 3 – Distribution of lesion areas at 12 weeks after MCAo, shown for seven rostrocaudal forebrain coronal levels in all groups. Treatment with hcG+EPO significantly reduced total lesion areas at two bregma levels compared to hcG+Saline treated groups. Values are mean \pm SEM, * p < 0.05, hcG+EPO vs. hcG+Saline.

employed a mitogen (epidermal growth factor) that has limited experience in human studies.

The current study aimed to replicate the findings of Kolb et al. (2007), but with two important changes geared toward promoting human translational application: using the IV/IM route instead of intraventricular, and replacing epidermal growth factor with a mitogen (hCG), which has a long history of safety in human subjects. In addition, a reduced dose of EPO (1400 U/kg per day during 3 days) was chosen to avoid unwanted side effects.

Poststroke recovery treatments are likely to enhance structural and functional reorganization (i.e. plasticity) of the damaged brain. Recent studies suggest that neurorestorative events, such as neurogenesis, angiogenesis, and synaptic plasticity, contribute to functional improvement after stroke (Chen and Chopp, 2006). Here we report that administration of hCG and EPO, together but not alone, stimulate tissue re-growth and recovery of motor function after transient MCAo. The improvement of behavioral score became evident only three weeks after ischemic insult and persisted throughout the 12-week survival period. Also this motor recovery was greater than that seen by any previous treatments on animals with similar functional loss (Kolb et al., 2007).

This study does not clarify the mechanism of this significant and substantial recovery. However, we speculate that several factors might contribute to the observations in this study. In terms of the progression of the injury, neuroprotective strategies are usually administered within hours of the time of injury in order to protect the ischemic core and progressive loss of neurological cells within the penumbra. Alternately, neuro-regeneration strategies should, in theory, have a much greater time window in which to administer therapeutics, ranging from hours to weeks. The

timing of the hCG and EPO effect of the regimen would argue in favor of the latter strategy: neuro-regeneration. However, although these compounds are administered for their primary neurogenic properties, hCG and EPO have well-described secondary pharmacologic effects that may contribute to improved structural and functional recovery, including angiogenic, anti-apoptotic, and anti-inflammatory actions, and perhaps even effects on brain plasticity.

There were several weaknesses apparent in the current study. Some of the secondary behavioral endpoints did not show the same effect of the treatment group that the primary behavioral endpoint did, possibly due to limited study power. Histological methods did not permit the same level of insight into the mechanism of treatment effect as compared to Kolb et al. (2007). Future studies will examine this issue in greater detail. In addition, we did not measure blood gases and plasma glucose in order to avoid the cannulation of the femoral artery, which often causes a hind limb ischemia and would be a problem in conducting four different behavioral tests. To control physiological parameters, the rats were orally intubated, immobilized with pancuronium bromide, and mechanically ventilated to avoid hypoxia. Rectal and cranial temperatures were monitored and held at normothermic levels throughout the experiment. One strength of the current study is that many experimental method details, such as timing and route of drug administration plus choice of medications, lay the groundwork for direct translation to human subjects with stroke (Clinicaltrials.gov identifier: NCT00362414). Another strength is the excellent safety record of hCG. Also, while safety of erythropoiesis stimulating agents has come under review, it should be noted that administration of EPO only during three days, and at submaximal doses, has generated a high level of confidence in the potential safety of the current approach.

4. Conclusions

Brain repair has the potential to improve the outcome in stroke patients. The current study found that both a two-drug and a one-drug approach, initiated 24 h after onset of experimental stroke in rats, improved behavioral outcome. The two-drug approach showed some advantages, particularly with respect to lesion volume. A clinical trial examining the safety of this approach in human subjects with stroke has been initiated (Clinicaltrials.gov identifier: NCT00362414).

5. Experimental procedures

5.1. Protocols and animal care

Experimental protocols were approved by the Inotek's Animal Care and Use Committee, Lexington, MA. Male Long Evans rats (280–330 g; Charles River Laboratories, Wilmington, MA) were fasted overnight but allowed free access to water. Following atropine sulfate (0.5 mg/kg, i.p.), anesthesia was induced with 3.5% halothane in a mixture of 70% nitrous oxide and a balance of oxygen. Rats were orally intubated, immobilized with pancuronium bromide (0.6 mg/kg, IV), and mechanically ventilated. Rectal (CMA/150 Temperature Controller, CMA/Microdialysis AB, Stockholm, Sweden) and cranial (left temporalis muscle) (Omega Engineering, Stamford, CT) temperatures were monitored and maintained at 36.0 ± 0.5 °C during surgical procedure. The right femoral vein was catheterized for drug infusion. During the 12-week survival period, rectal temperature and body weight were monitored periodically.

5.2. Middle cerebral artery occlusion

The right middle cerebral artery (MCA) was occluded for 90 min by a modification (Belayev et al., 1996) of the intraluminal-suture occlusion method (Longa et al., 1989). In brief, the right common carotid artery (CCA) was exposed through a midline neck incision and dissected free of surrounding nerves. The occipital branches of the external carotid artery (ECA) were coagulated, and the pterygopalatine artery was ligated. A 4-cm length of 3-0 monofilament nylon suture was then inserted via the proximal ECA into the internal carotid artery (ICA) and MCA, a distance of 20–22 mm from the CCA bifurcation according to the animal's weight, thereby occluding the MCA. Prior to use, the tip of the suture was heat-blunted, and a 22-mm distal segment of the suture was coated with poly-L-lysine solution (0.1% [wt/vol]) and dried at 60 °C for 1 h; this coating procedure enhances the reproducibility of the resulting infarct (Belayev et al., 1996). After suture placement, the neck incision was closed, and animals were allowed to awaken from anesthesia. At 60 min following MCA occlusion (MCAo), animals were tested on a standardized neurobehavioral battery to confirm the presence of a neurological deficit (Belayev et al., 1996). After 90 min of MCAo, rats were reanesthetized, temperature probes were reinserted, and the intraluminal suture was carefully removed.

After surgery rats were observed carefully for signs of discomfort; no such signs were observed. Animals were housed in individual cages.

5.3. Behavioral assessments

The primary behavioral endpoint was the Composite Neurological Score (0–12 points, 0=normal and 12=maximal deficit) (Belayev et al., 1996), a battery that consists of two tests that have been used previously to evaluate various aspects of neurologic function: (1) the postural reflex test, developed by Bederson et al. (1986) to examine upper body posture while the animal is suspended by the tail; and (2) the forelimb placing test, developed by De Ryck et al. (1989) to examine sensorimotor integration in forelimb placing responses to visual, tactile and proprioceptive stimuli. Tests were conducted by an observer blinded to the treatment group. The composite neurological score was tested 1 h into the MCAo, then again at 24 h, 48 h, 1 wk, 2 wk, 3 wk, 4 wk, 6 wk, 8 wk, 10 wk, and 12 wk post-MCAo. The severity of stroke injury was assessed by behavioral examination of each rat at 60 min after onset of MCAo. Rats that did not demonstrate high-grade contralateral deficit (score, 10–11) were excluded from further study. Three secondary behavioral endpoints were also measured, each eight times after stroke, starting at week 1, 2, 3, 4, 6, 8, 10, and 12 post-MCAo. These endpoints were as follows: (1) adhesive test (Schallert et al., 2003), which measures sensory function during 2 min, with four trials per day, and each trial separated by at least 5 min; (2) forepaw inhibition (swimming) test (Gonzalez et al., 2003), which is an indicator of forelimb impairment. Scoring of forelimb inhibition is done by counting the numbers of strokes made by each forelimb, and reported as (strokes made with impaired limb)–(strokes made with nonimpaired limb) (five trials were performed per day); (3) cylinder test (Jones et al., 2003), which measures abnormalities of forelimb postural-motor behavior. The forelimb asymmetry score was calculated using the formula: $(\text{total ipsilateral limb use} + 1/2 \text{ bilateral}) / \text{total limb use} \times 100$. Each animal was videotaped for 5 min.

5.4. Treatment and experimental groups

The two active drugs were human chorionic gonadotropin (hCG, Harbor-UCLA Research and Education Institute, Torrance, CA), given as 440 IU/day, IM, on days 1, 3, and 5; and erythropoietin (EPO, Epogen, Henry Schein, Melville, NY), given as 1440 IU/day, IV, by an osmotic pump (Alzet 2ML1; Cupertino, CA) into the right femoral vein, on days 6, 7, and 8 after MCAo. In the current study, hCG was used as a mitogen for endogenous neural stem cells for two reasons. First, our data (unpublished observations) found hCG to be an effective mitogen. Second, most of the factors identified in prior studies as having endogenous neural stem cells mitogen activity have limited clinical experience in humans, while hCG is known to have an excellent safety profile.

At 24 h after MCAo, the animals were randomly assigned to one of four treatment groups: Group A (hCG+EPO, $n=13$), Group B (hCG+Saline, $n=15$), Group C (Saline+EPO, $n=12$) and Group D (Saline+Saline, $n=13$). The treatment schedule and methods were identical across the four groups.

5.5. Histopathology

Following a 12-week survival period, animals were deeply anesthetized with halothane and perfused transcardially with isotonic saline followed by a perfusion with paraformaldehyde (4% in phosphate buffer). Brains were then removed and brain blocks were embedded in paraffin. Twelve-micron-thick sections were cut in the coronal plane and stained with hematoxylin and eosin. Lesion volume and percent normal tissue loss were quantitated by digitizing histological sections (MCID™ Core imaging software, InterFocus Imaging Ltd, Linton, Cambridge, UK) at 7 standardized coronal levels (bregma levels: +2.7, +1.2, −0.3, −1.3, −1.8, −3.8 and −5.0 mm) (Konig and Klippel, 1963). An investigator blinded to the experimental groups outlined the areas of the lesion, which were clearly demarcated; the ventricles, as well as the left and right hemisphere, were contoured at each level. The following analysis was conducted: (A) Lesion volume was calculated as the product of the cross-sectional area for all sections, and the distance between the sections was determined using Simpson's method (Carnevale 1986). (B) Residual (normal) tissue in the right hemisphere (mm³) was calculated applying the following formula: (right hemisphere volume − right ventricle − lesion volume). (C) Tissue loss was calculated as a difference in the amount of histologically-intact residual tissue between the lesioned and the unlesioned hemispheres. (D) Percent [relative to unlesioned (left) hemisphere volume] was calculated applying the following formula: (Tissue loss × 100 / Residual tissue in left hemisphere).

5.6. Statistical analysis

Data were presented as mean values ± SEM. Neurobehavioral scores and infarct size data were analyzed by repeated-measures analysis of variance (ANOVA) with post hoc tests. Physiological variables were compared using Student t tests. Differences at $p < 0.05$ were considered statistically significant.

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